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Tilletia puccinelliae, a new species of reticulate-spored bunt fungus infecting *Puccinellia distans*

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Abstract: A shipment of Fults alkaligrass seed (*Puccinellia distans*) grown in Washington state containing bunted florets was intercepted by quarantine officials at China's Tianjin Entry-Exit Quarantine and Inspection Bureau. The bunted florets were filled with irregularly shaped, reticulately ornamented teliospores that germinated in a manner characteristic of systemically infecting *Tilletia* spp. on grass hosts in subfamily Pooideae. Based on morphological characters and a multigene phylogenetic analysis of the ITS region rDNA, eukaryotic translation elongation factor 1 alpha and a region of the second largest subunit of RNA polymerase II including a putative intein, the *Puccinellia* bunt is genetically distinct from known species of *Tilletia* and is proposed as a new species, *T. puccinelliae*.

Key words: alkaligrass, cool season grass smuts, multigene phylogenetic analysis, Tilletiales

INTRODUCTION

In 2004 bunted florets (smut sori) were found in a shipment of U.S. Fults alkaligrass seed, *Puccinellia distans* (Pooideae, Poaceae), by scientists at the Tianjin Entry-Exit Quarantine and Inspection Bureau in Tanggu, China. The bunted florets were filled with teliospores with ornamentation and germination characteristic of *Tilletia* (Ustilaginomycotina, Basidiomycota). None of the approximately 150 species recognized in *Tilletia* is known to infect *Puccinellia* spp.; the only smuts reported on *Puccinellia* are species of *Entyloma*, *Urocystis* and *Ustilago* (Durán and Fischer 1961, Fischer 1953, Vánky 1994, Guo and Zhang 2004). However two grassy weeds present in alkaligrass seed fields, *Apera interrupta* (windgrass) and *Bromus tectorum* (downy brome, cheatgrass) respectively are hosts for *Tilletia goloskokovii* and *T. bromi* (Boyd and Carris 1997, 1998; Boyd et al. 1998). The teliospores of *T. bromi* and *T. goloskokovii* are morphologically similar to each other (Boyd et al. 1998) and to the spores in the bunted florets in the Fults alkaligrass seed.

A multigene phylogenetic analysis of the *Puccinellia* bunt, *T. bromi*, *T. goloskokovii* and similar species on hosts in subfamily Pooideae was conducted. Results of the analyses and comparison of morphological characters of *Tilletia* spp. associated with cool season grass seed crops in Pacific Northwest USA are presented.

MATERIALS AND METHODS

Isolation, maintenance and deposition of cultures and voucher specimens.—Species used in this study are listed (TABLE I). Host names and authorities are listed as in the U.S. Department of Agriculture PLANTS Database (http://plants.usda.gov). Voucher materials were deposited in herbarium WSP (Washington State University, Pullman, Washington), herbarium BPI (Beltsville, Maryland) and herbarium H.U.V. (Tübingen, Germany). Representative cultures were deposited in Centraalbureau voor Schimmelcultures (CBS; Utrecht, the Netherlands) (see TABLE I). Seed samples 25–430 g from 10 seed lots of Fults alkaligrass grown in Franklin County, Washington in 2002 (one sample) and 2004 (nine samples) were examined with a modification of the seed-wash protocol used for the US National Karnal Bunt Survey (Peterson et al. 2000). Twenty-five grams seed in 100 mL tap water with two drops of Tween-20 were placed in a 500 mL flask on a rotary shaker at 200 revolutions/min for 10 min. Seed suspension was passed through a 53 μm pore sieve into a clean 600 mL beaker. The suspension from the beaker was poured through a 25 μm pore sieve, and any spores and debris remaining on the sieve were washed in a 15 mL conical centrifuge tube and pelleted by centrifugation 3 min at 200 revolutions/min. The supernatant was discarded and the pellet was suspended in 100 μL Shear's mounting medium (50 mL 2% [w/v] potassium acetate, 20 mL glycerol, 30 mL 95% ethyl alcohol). The suspension was transferred to microscope slides, a 22 × 50 mm cover slip was placed on each slide, and the slides were examined at 125× with a compound microscope. If reticulately ornamented teliospores were observed on the slides, seeds were soaked in

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TABLE I. Isolates and DNA sequences used in this study

Taxon	Voucher/culture numbers	Year	Host	Origin	Collector	GenBank accession numbers		
						ITS1	RPB2	EF1A
Tilletia brevifaciens G.W. Fischer	H.U.V. 20.802	2004	*Thinopyrum intermedium* Barkworth & D. R. Dewey × *Elymus repens* (L.) Gould	Poland	K. Vánky	EU257565[a]	EU257599[a]	EU257535[a]
T. brevifaciens	WSP 68945 (Vánky 412)	1999	*Thinopyrum intermedium*	Austria	K. Vánky	EU257566[a]	EU257600[a]	EU257536[a]
T. bromi (Brockmuller) Brockmuller	WSP 71271 (LMC 148)	1992	*Bromus tectorum* L.	US (Washington)	L. Carris	EU257555[a]	EU257592[a]	EU257528[a]
T. bromi	WSP 71272 (LMC 167; CBS 123002)	1992	*Bromus arvensis* L.	US (Wyoming)	L. Carris	EU257556[a]	EU257593[a]	EU257529[a]
T. bromi	WSP 71273 (LMC 75; CBS 123001)	1991	*Bromus hordeaceus* L. ssp. *hordeaceus*	US (Wyoming)	L. Carris	EU257557[a]	EU257594[a]	EU257530[a]
T. caries (DC.) Tul.	WSP 71304 (DAR 73302)	1997–1999	*Triticum aestivum* L.	Australia	G. Murray	EU257559[a]	EU257596[a]	EU257532[a]
T. caries	WSP 71303 (LMC J-19; CBS 121951)	1995	*Triticum aestivum*	Sweden	L. Johnsson	EU257560[a]	EU257597[a]	EU257533[a]
T. contraversa Kuhn	WSP 71280 (LMC 282; CBS 121952)	1994	*Triticum aestivum*	US (Oregon)	L. Carris	EU257561[a]	EU257598[a]	EU257534[a]
T. contraversa	WSP 69062 (Vánky 528)	1984	*Triticum aestivum*	Germany	K. Vánky	EU257562[a]	EU257588[a]	EU257526[a]
T. elymi Diet. & Holw.	WSP 71274 (LMC 158; CBS 123000)	1992	*Elymus glaucus* Buckl. ssp. *glaucus*	US (Wyoming)	L. Carris	EU257564[a]	EU257591[a]	EU257527[a]
T. fusca Ell. & Everh.	WSP 71275 (LMC 141; CBS 122991)	1971	*Vulpia microstachys* (Nutt.) Munro var. *microstachys*	US (Washington)	L. Carris	EU257567[a]	EU257601[a]	EU257537[a]
T. goloskokovii	WSP 69687 (LMC 315; CBS 122995)	1995	*Apera interrupta* (L.) Beauv.	US (Washington)	L. Carris	EU257569[a]	EU257603[a]	EU257539[a]
T. goloskokovii	WSP 71281 (LMC 238-2)	1993	*Apera interrupta*	US (Washington)	L. Carris	EU257568[a]	EU257602[a]	EU257538[a]
T. laevis Kuhn	WSP 71278 (LMC178)	1971	*Triticum aestivum*	US	L. Carris	EU257571[a]	EU257605[a]	EU257541[a]
T. laevis	WSP 71300 (Vánky 766; CBS 121950)	1988	*Triticum aestivum*	Iran	K. Vánky	EU257573[a]	EU257607[a]	EU257543[a]
T. laguri G.M. Zhang, G. X. Lin & J. R. Deng	HUV 16.352	1992	*Lagurus ovatus* L.	Italy	K. Vánky	EU257574[a]	EU257608[a]	
T. lolii Auers.ex Rab.	WSP 71298 (Vánky 767)	1990	*Lolium rigidum* Gaudin	Iran	K. Vánky	EU257575[a]	EU257609[a]	EU257544[a]
T. lolioli Vánky, Carris, Castl. & H. Scholz.	WSP 71305 (Vánky 763)	1990	*Loliolum subulatum* (Banks & Sol.) Eig	Iran	K. Vánky	EU257576[a]	EU257610[a]	EU257545[a]
T. puccinelliae Carris, G. Huang & Castl.	WSP 71469 HOLOTYPE (NBS HF4 FUL3-2 205 P7; ex-type CBS 122993)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910057[b]	EU910063[b]	EU910051[b]

TABLE I. Continued.

Taxon	Voucher/culture numbers	Year	Host	Origin	Collector	GenBank accession numbers		
						ITS1	RPB2	EF1A
T. puccinelliae	WSP 71465 (NBS HF4 FUL3 seed 2; CBS 122994)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910058[b]	EU910064[b]	EU910052[b]
T. puccinelliae	WSP 71472 (NBS NE4 FUL 1-2 palea/lemma; CBS 122996)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910059[b]	EU910065[b]	EU910053[b]
T. puccinelliae	WSP 71470 (NBS NE4 FUL 4-6 sm intact; CBS 122997)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910060[b]	EU910066[b]	EU910054[b]
T. puccinelliae	WSP 71470 (NBS NE4 FUL4-1 lg broken; CBS 122998)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910061[b]	EU910067[b]	EU910055[b]
T. puccinelliae	WSP 71471 (LH52 425B-9; CBS 122999)	2004	*Puccinellia distans*	US (Washington)	L. Carris	EU910056[b]	EU910062[b]	EU910050[b]
T. secalis (Cda.) Koern.	WSP 71279 (LMC 255)	1993	*Secale cereale* L.	US (Idaho)	L. Carris	EU257577[a]	EU257589[a]	EU257525[a]
T. sphaerococca (Rab.) Fisch. v Waldh.	culture	2003	*Agrostis stolonifera* L.	US (Chinese Intercept)	G. Huang	EU257578[a]	EU257611[a]	EU257546[a]
T. togwateei	WSP 71277 (LMC 169)	1992	*Poa reflexa* Vasey & Scribn. ex Vasey	US (Wyoming)	L. Carris	EU257580[a]	EU257613[a]	EU257547[a]
T. trabutii Jacz.	WSP 71299 (Vánky 764; CBS 122324)	1990	*Hordeum murinum* L. ssp. *glaucum* (Steud.) Tzvlev	Iran	K. Vánky	EU257581[a]	EU257614[a]	EU257548[a]
T. trabutii	VPRI 32106	2005	*Hordeum murinum* L. ssp. *leporinum* (Link) Arcang.	Australia	I. Pascoe	EU257582[a]	EU257615[a]	EU257549[a]
T. vankyi Carris & Castlebury	WSP 71266 (V21-713)	1997	*Lolium perenne* L.	Australia	L. Carris	EU257587[a]	EU257620[a]	EU257554[a]
T. vankyi	WSP 71270 (FF1-1; CBS 122323)	2005	*Festuca rubra* L. ssp. *fallax* (Thuill.) Nyman	US (Oregon)	S. Alderman	EU257585[a]	EU257618[a]	EU257552[a]

[a] Cited from Carris et al. 2007.
[b] From this study.

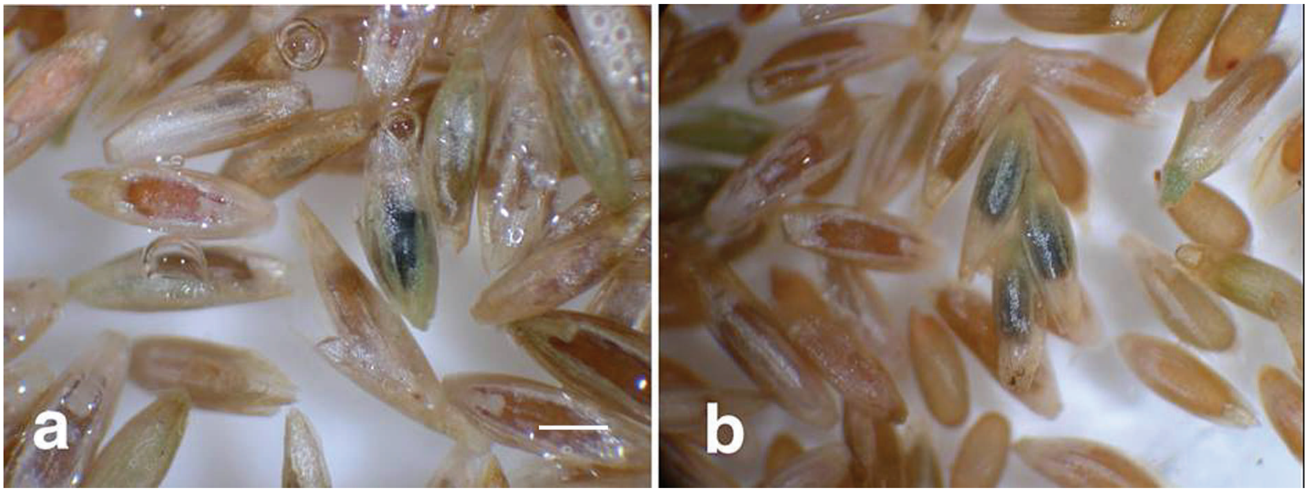

FIG. 1a, b. *Tilletia puccinelliae* (WSP 71469, holotype) sori in seed of *Puccinellia distans*; seed soaked overnight in water to render palea and lemma transparent. a. Single bunted floret. b. Three bunted florets attached to rachis. Bar = 1 mm.

water overnight to render the palea and lemma translucent and were examined under low power (10×) magnification to detect bunted florets (FIG. 1). Teliospores from bunted florets were mounted in Shear's mounting medium and examined with differential interference contrast microscopy at 1000×. Teliospore diameter, including exospore, shape, color, thickness of exospore, number of meshes per spore diameter, and sterile cell shape, color and thickness were recorded for 20 spores per sample (TABLE II). For germination teliospores were surface-sterilized in 0.26% NaClO (5% commercial bleach) in a 1.5 mL Eppendorf tube for 50 s, pelleted by centrifugation at approximately 13 000 *g* in a benchtop microcentrifuge for 10 s and rinsed twice with sterile, distilled water. Surface-sterilized teliospores were streaked on 1.5% water agar (WA) and incubated at 5, 15 C and room temperature (20–25 C). Primary basidiospores were transferred to M-19 agar (Trione 1964) to establish colonies for nucleic acid extraction. Cultures on M-19 were maintained at 15 C in the dark.

Nucleic acid extraction and PCR amplification.—DNA was extracted directly from actively growing surface mycelium scraped from M-19 plates. DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California), according to the manufacturer's instructions, with approximately 50 mg fresh mycelium.

All amplifications were performed in 20 μL aliquots on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California). Three PCR-amplified nuclear DNA fragments, elongation factor 1α (EF1A), internal transcribed spacer 1 (ITS1) and the second largest subunit of RNA polymerase II (RPB2) were used as described by Carris et al. (2007). The RPB2 region amplified was shown to be informative for phylogenetic studies of Tilletiales and is more variable in sequence than EF1A and ITS1 regions (Carris et al. 2007). Recent analysis discovered motif similarity between the RPB2 amplified regions from Tilletiales and other fungal intein coding regions (Poulter et al. 2007 unpubl). The primers were used with these combinations: (i) EF1-526F with EF1-1567R (Rehner, S. 2001. Primers for elongation factor 1-a (EF1-a). http://ocid.nacse.org/research/deephyphae/EF1primer.pdf.); (ii) ITS5 or ITS1 with ITS4 (White et al. 1990) as the reverse primer; (iii) RPB2-740F and RPB2-1365R (Carris et al. 2007).

PCR products were purified with ExoSAP-IT (USB, Cleveland, Ohio) according to the manufacturer's instructions and amplified with respective forward and reverse PCR primers with the BigDye 3.1 dye terminator kit (Applied Biosystems, Foster City, California). Those products were purified again with Performa DTR gel filtration cartridges (Edge BioSystems, Gaithersburg, Maryland) and sequenced on an ABI 3100 automated DNA sequencer.

Sequence analysis.—The chromatographic outputs were assembled and edited into consensus sequences with Sequencher 4.5 for Windows (Gene Codes Corp., Ann Arbor, Michigan). Eighteen sequences of three regions from six isolates were generated, which were aligned individually to alignments of 21 isolates from Carris et al. (2007), with Clustal W as implemented in BIOEDIT 7.0.5.3 for Windows (Hall 1999). The individual gene alignments were edited manually in BIOEDIT and concatenated to produce the final multiple-gene dataset. All gaps in the dataset were treated as missing data.

Two methods were used to test for conflict among the genes, the partition homogeneity test (incongruence length difference test) as implemented in PAUP (Swofford 2002) with 1000 replicates and a reciprocal bootstrap analysis (Reeb et al. 2004). Independently inferred topologies from each partition were examined for conflict (e.g. one monophyletic and the other paraphyletic). Reciprocal 80% ML bootstrap (ML-BS) value or 95% Bayesian posterior probability (PP) were used as criteria for strongly supported clades.

Phylogenetic trees were inferred independently from each partition and the combined alignment by maximum likelihood (ML) with PAUP 4.0b10 as well as Bayesian method with MrBayes 3.1.2 for Windows (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). DNA substitution models were determined by Modeltest 3.7 (Posada and Crandall 1998). The best fit models and parameters were chosen with Akaile information criterion (AIC) and hierarchical likelihood ratio tests (hLRT).

For ML analysis heuristic search was chosen with 100 starting trees obtained via stepwise, random addition of sequences. The branch-swapping algorithm is tree bisection-reconnection (TBR) on each tree. All aligned positions were included in the analysis. All characters are unordered and have equal weight. Specific substitution models determined by Modeltest were used for each dataset as follows: (i) EF1A, TrN + I + G model with Base = (0.2338 0.3171 0.2555), Nst = 6, Rmat = (1.0000 1.6571 1.0000 1.0000 9.3667), Rates = gamma, Shape = 0.7539, Pinvar = 0.5776; (ii) ITS1, TVM + I model with Base = (0.2443 0.2249 0.2155), Nst = 6, Rmat = (4.9187 4.9305 0.4291 0.6961 4.9305), Rates = equal, Pinvar = 0.9013; (iii) RPB2, TrN + G model with Base = (0.1801 0.2732 0.3165), Nst = 6, Rmat = (1.0000 4.1486 1.0000 1.0000 6.1422), Rates = gamma, Shape = 0.1834, Pinvar = 0; (iv) EF1A + ITS1 + RPB2, TrN + I + G model with Base = (0.2190 0.2754 0.2614), Nst = 6, Rmat = (1.0000 3.0572 1.0000 1.0000 6.7569), Rates = gamma, Shape = 0.7073, Pinvar = 0.6948. Branch support was estimated using nonparametric bootstrap analysis with 1000 replicated samples. Majority rule consensus trees were generated with ML bootstrap values higher than or equal to 80%.

The Bayesian inference of phylogeny was performed with Monte Carlo Markov chain (MCMC) sampling. Several GTR models without parameters were used as the priors in MrBayes. The priors for each datasets were: (i) EF1A = GTR + I + G; (ii) ITS1 = GTR + I; (iii) RPB2 = GTR + G; (iv) the combined gene was unlinked in Statefreq, Revmat, Shape and Pinvar so that each partition (gene) could be applied with the corresponding GTR model described above. MCMC sampling was limited to 500 000 generations. Two independent runs were started simultaneously, with four chains in each run (one heat chain and three cold chains). The frequency of swap was set to 1. Trees were sampled every 100 generations. The first 25% sampled trees were excluded from the analysis. Well supported clades were inferred with a minimum of 95% Bayesian posterior probabilities.

RESULTS

Seed survey.—Reticulately ornamented teliospores were found in seed washes for nine of the 10 seed samples available from 2004. Number of teliospores ranged from 9 to approximately 3500 per 25 g seed. No teliospores were found in the 2002 sample. The number of bunted florets per 25 g seed in the samples in which teliospores were present ranged from 0 (with 9 and 34 teliospores/25 g seed) to 121 (3500 teliospores/25 g seed). All bunted florets were filled with teliospores, and several remnants of inflorescences with 2–3 bunted florets attached to the rachis were found (FIGS. 1, 2)

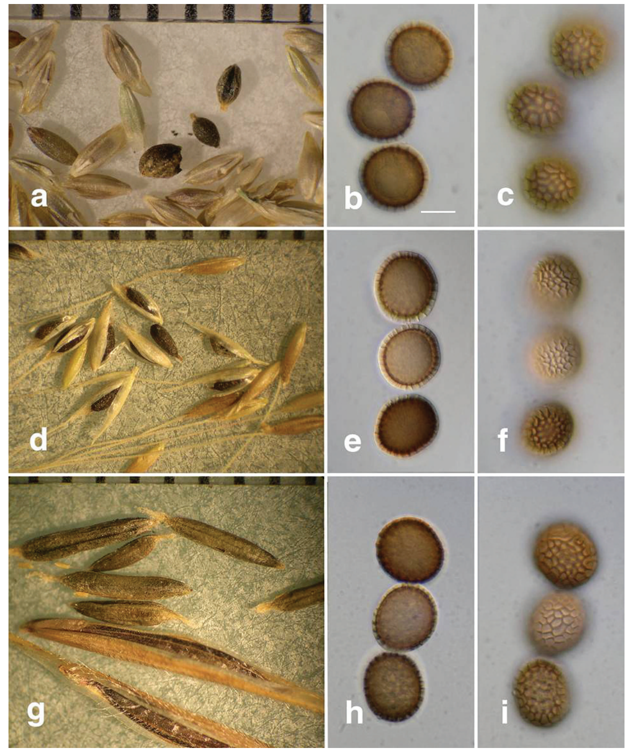

FIG. 2. *Tilletia* spp. associated with seed of *Puccinellia distans*. a–c. *T. puccinelliae*. a. Bunted and healthy florets of *Puccinellia distans*. b. Teliospores, plane view. c. Teliospores, surface view of reticulate ornamentation. d–f. *T. goloskokovii*. d. Bunted and healthy florets of *Apera interrupta*. e. Teliospores, plane view. f. Teliospores, surface view of reticulate ornamentation. g–i. *T. bromi*. g. Bunted and healthy florets of *Bromus tectorum*. h. Teliospores, plane view. i. Teliospores, surface view of reticulate ornamentation. Units a, d, g = 1 mm; bar b, c, e, f, h, i = 10 μm.

Morphology.—Morphological and germination characters for the *Puccinellia* bunt fungus and four other *Tilletia* spp. that either infect cultivated cool season grasses in the Pacific Northwest USA (*T. sphaerococca* and *T. vankyi*) or are likely to occur as contaminants in *P. distans* based on the known distribution of their hosts (*T. bromi* and *T. goloskokovii*) are summarized (TABLE II).

Phylogenetic analysis.—The combined alignment consisted of EF1A (721 bp), ITS1 (649 bp) and RPB2 (587 bp), with a total of 1957 characters. Of those characters 1724 are constant, 86 are variable but parsimony uninformative and 147 are parsimony informative. A ML tree was inferred by combined analysis of all three loci (FIG. 4). A clade containing all isolates of the *Puccinellia* bunt was identified from the ML tree (ML-BS: 94%, PP: 95%). The *Puccinellia* bunt

TABLE II. Host, infection type, morphology and germination of *Tilletia* species compared in this study

	Tilletia puccinelliae [b]	*Tilletia bromi* [a]	*Tilletia goloskokovii* [a]	*Tilletia sphaerococca* [a]	*Tilletia vankyi* [a]
Hosts	*Puccinellia distans*	*Bromus tectorum*	*Apera interrupta*	*Agrostis stolonifera*	*Lolium perenne, L. multiflorum, Festuca rubra*
Infection type	Systemic	Systemic	Systemic	Systemic	Local?
Sori (mm)	1–1.5 × 0.5–0.8	1.5–4 × 0.5–1	1–1.5 × 0.5–0.75	0.75–1 × 0.3–0.5	1.5–3 × 1–1.5
Teliospores:					
Diameter (μm)	18.5–31 × 15–28	17–24.5	17.5–30	21–30	17.5–30
Exospore depth (μm)	0.5–1.5(–2)	1–2	1–3	1.5–3.5	1–2
Meshes/spore diam	6–10 (–14)	7–9	4–8	5–10	5–8
Color	Medium to dark reddish brown	Medium to dark reddish brown	Pale to dark fuscous brown	Pale yellow-brown to dark brown	Pale to medium reddish brown
Shape	Subglobose to angular	Globose	Globose	Globose	Globose
Germination	5 C (10–12 d)	5–15 C (7–21 d)	5–10 C (7–24 d)	5 C (22–30 d)	5 C (> 14 d), 15 C (5–7 d)
Sterile cells:					
Diameter (μm)	11–17.5 × 10.5–16.5	10.5–23	15–28	9.5–23	10–21
Wall (μm)	1–2.5	1–2.5	1–2.5	1–2.5	0.5–2
Primary basidiospores:					
Shape	Filiform	Filiform	Filiform	Filiform	Filiform
Number/basidium	9–16	9–18	22–26	26–32	24–40
Size (μm)	70–80 × 2.5	57–88 × 2–2.5	48.5–68 × 2–2.5	68.5–75.5 × 2–2.5	53–74 × 1.5–3.5
# Nuclei/ basidiospore	1	1	1	1	1
Conjugation	Yes	Yes	Yes	Yes	No

[a] Cited from Carris et al. 2007.
[b] From this study.

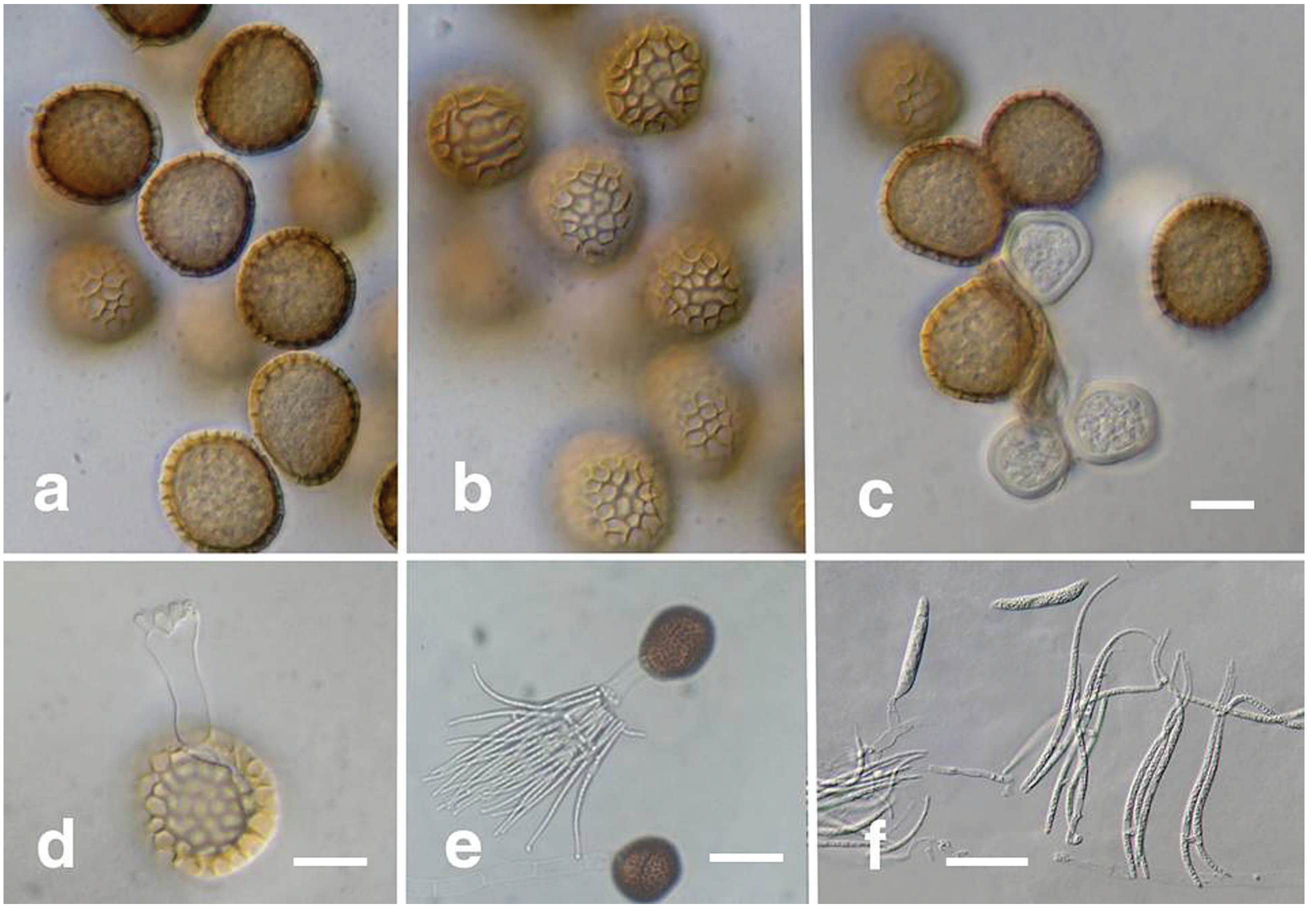

FIG. 3. *Tilletia puccinelliae* (WSP 71469, holotype). a. Teliospores, plane view. b. Teliospores, surface view of reticulate ornamentation. c. Teliospores and sterile cells. d. Teliospore and basidium. e. Germinated teliospore and conjugated basidiospores. f. Conjugated basidiospores and sporidia (secondary basidiospores). Bar a–c = 10 μm; d = 10 μm; e = 20 μm; f = 20 μm.

was distinct but with 86% ML-BS support grouped with two isolates of *T. bromi* isolates in a well supported clade (ML-BS: 100%, PP: 100%). Other well supported clades include those containing isolates of *T. vankyi* (ML-BS: 99%, PP: 100%), *T. brevifaciens* (ML-BS: 96%, PP: 100%), *T. goloskokovii* (ML-BS: 99%, PP: 100%). Isolates of three wheat bunt pathogens, *T. contraversa*, *T. caries* and *T. laevis*, form a well supported clade (ML-BS: 83%, PP: 100%) but without internal support to distinguish individual species.

Incongruence among the three genes was evaluated by partition homogeneity test in PAUP. A strong conflict was indicated ($P = 0.001$ for all genes). One source of incongruence is that the ML tree topologies inferred by the ITS1 locus included six different trees with the same likelihood score. Incongruence of tree topologies for the three genes also was examined by looking for conflict among well supported clades. In general clades inferred from EF1A and ITS genes were not as well supported as those from RPB2. Among well supported clades (80% ML-BS and 95% PP) none were found to be in conflict. In the clades with either 80% ML-BS or 95% PP incongruence was found in the placement of *T. secalis*, which was clustered with two *T. brevifaciens* isolates by EF1A gene (ML-BS: 100%, PP: 100%), while clustered with *T. trabutii*, *T. goloskokovii*, *T. sphaerococca* and *T. lolioli* by the RPB2 gene (ML-BS: 47%, PP: 99%) (trees not shown). This result is consistent with results of Carris et al. (2007), and *T. secalis* was not excluded from the analyses in this study. The sequence alignment and phylogenetic trees were deposited in TreeBASE as SN4002.

TAXONOMY

Tilletia puccinelliae Carris, Castl. & G. Huang, sp. nov. FIGS. 1, 2a–c, 3

MycoBank MB 512086

Etymology. derived from the host plant *Puccinellia*.

Sori in ovario inclusi, ellipsoidei-citriformes, 1–1.5 × 0.5–0.8 mm; massa sporarum foetida, fusci brunneo et pulverulenta. Cellulae steriles subglobosae vel irregulares, 11–17.5 × 10.5–16.5 µm, hyalineae, cum parietibus 1–2.5 µm crasso, levibus. Sporae globosae, subglobosae, ovoidae, ellipsoidales, rotundae subpolyedrice irregulars, plerumque cum complanatus lateribus, usque elongatae, raro cum subacutae apice, subrubeo-brunneae, 18.5–31 × 15–28 µm, exosporium subtiliter reticulatum usque incompletae reticulatum, raro cerebriformae, muri 0.5–1.5(–2) µm alti, 6–10(–14) in circulo aequatoriali. Basidiosporae filiformae, 9–16 per basidium, conjugatae, uninucleatae, 70–80 × 2.5 µm.

Sori in ovaries of *Puccinellia distans*, enclosed by pericarp, ellipsoidal to lemon-shaped, 1–1.5 × 0.5–0.8 mm; spore mass foetid, dark fuscous brown, powdery (FIGS. 1, 2a). Sterile cells subglobose to irregular, 11–17.5 × 10.5–16.5 µm, hyaline, wall 1–2.5 µm thick, smooth (FIG. 3c). Teliospores globose, subglobose, ovoid, ellipsoidal, irregularly rounded subpolyhedral, often with a flattened side, to elongated, rarely with a subacute tip, medium reddish-brown, close to chestnut (40, Rayner 1970), 18.5–31 × 15–28 µm, exospore finely reticulate to incompletely reticulate, rarely cerebriform (Vánky 1991), muri 0.5–1.5 (–2)µm high, with 6–10(–14) meshes per spore diameter (FIGS. 2b, c, 3a–c). Teliospore germination 10–12 d at 5 C on WA; no germination at room temperature and 15 C. Basidium simple, up to 80 µm long (FIG. 3d, e); basidiospores 9–16 per basidium, conjugating, uninucleate, hyaline, filiform, 70–80 × 2.5 µm (FIG. 3e, f). Sporidia (ballistospores) allantoid, 26–35 × 4.5–6 µm, formed from subulate sporogenous cells formed from hyphae or primary basidiospores (FIG. 3f).

Holotype.—USA: Washington, Franklin County: ovaries of *Puccinellia distans*, 2004, L.M. Carris WSP 71469. *Isotypes*: BPI 878741, HUV 20962. *Ex-type*: CBS 122993.

Additional representative collections examined.—TABLE I.

Commentary.—Among the *Tilletia* spp. most likely to occur as contaminants in cool season grass seed, the irregular shape of the teliospores in *T. puccinelliae* is a distinctive feature of this species, but the size, color and ornamentation of the teliospores are similar to *T. bromi* and *T. goloskokovii*. The sterile cells of *T. puccinelliae* are smaller than those of *T. bromi* and *T. goloskokovii*, approximately half the diameter of the teliospores (TABLE II, FIG. 3a–c). The size and shape of primary basidiospores of the five species (compared in TABLE II) overlap, but the number of basidiospores per basidium in the *Puccinellia* bunt (9–16) is more similar to *T. bromi* than *T. goloskokovii*. The sori of *T. puccinelliae* are similar in size and shape to those of *T. goloskokovii* (FIG. 2a, b). *Tilletia bromi* sori (FIG. 2c) in contrast are up to three times as long as those of either *T. goloskokovii* (FIG. 2b) or *T. puccinelliae* (FIG. 2a).

DISCUSSION

Genus *Puccinellia* Parl. belongs in tribe Poeae of the grass subfamily Pooideae and is most closely related to *Poa* and *Sclerochloa* among the festucoid grass genera (Catalán et al. 2004). There are no known smuts on *Sclerochloa*. Six *Tilletia* species are reported to infect *Poa*, *T. cathcartae* Durán & G.W. Fisch., *T. paradoxa* Jacz., *T. poae* Nagorny, *T. sterilis* Ule, *T. togwateei* and *T. transiliensis* M.N. Kusnezow & Schwarzman. Teliospores of *T. paradoxa*, *T. transiliensis* and *T. cathcartae* have echinulate or tuberculate ornamentation, and sori of *T. sterilis* form in leaf sheaths and culms (Durán and Fischer 1961). Teliospores of *T. poae* are reticulate, similar in size to those of *T. puccinelliae* but more regular in shape and with deeper exospore than those of *T. puccinelliae* (Vánky 1994); no records of *T. poae* were found for North America. *T. togwateei*, an ovary-infecting species with reticulate spores, is included in the present analysis (FIG. 4). *T. puccinelliae* is easily distinguished from other genera of smuts on *Puccinellia* spp. For example three species of *Ustilago*, *U. hypodytes* (D.F.L. Schlechtendal) E. Fries, *U. spegazzinii* Hirschh. and *U. trebouxii* H. & P. Sydow, have been reported from North America (Fischer 1953), all of which form sori in the leaves and leaf sheaths of *P. distans*. *Entyloma dactylidis* (G. Passerini) R. Ciferri, the only known species in this genus on *Puccinellia*, forms sori in the leaves and leaf sheaths with densely packed, smooth teliospores (Vánky 1994). *Urocystis puccinelliae* L.Guo & H.C. Zhang (2004) is reported on *Puccinellia tenuiflora* (Griseb.) Scribn. & Merr. from China. Species of *Urocystis* are readily distinguished from species of *Tilletia* by formation of spore balls in sori.

The ca. 25 species in *Puccinellia* occur in saline soils in coastal and inland habitats (Choo et al. 1994). *P. distans* is a Eurasian perennial species that has become established throughout much of North America (Hitchcock 1950). Increased salinity due to the use of effluent and low quality water for irrigation of golf courses and other types of turf has resulted in a demand for salt-tolerant turfgrasses, and Fults alkaligrass is one of the most salt-tolerant cool season grasses (Alshammary et al. 2004). Approximately 121 ha Fults alkaligrass are grown for seed production in Washington. *Tilletia puccinelliae* is known only from individual bunted florets detected in eight seedlots of alkaligrass cv. Fults from Franklin County, Washington, produced during the 2004 growing season. Infected plants have not been observed in the field, and the manner in which *T. puccinelliae*

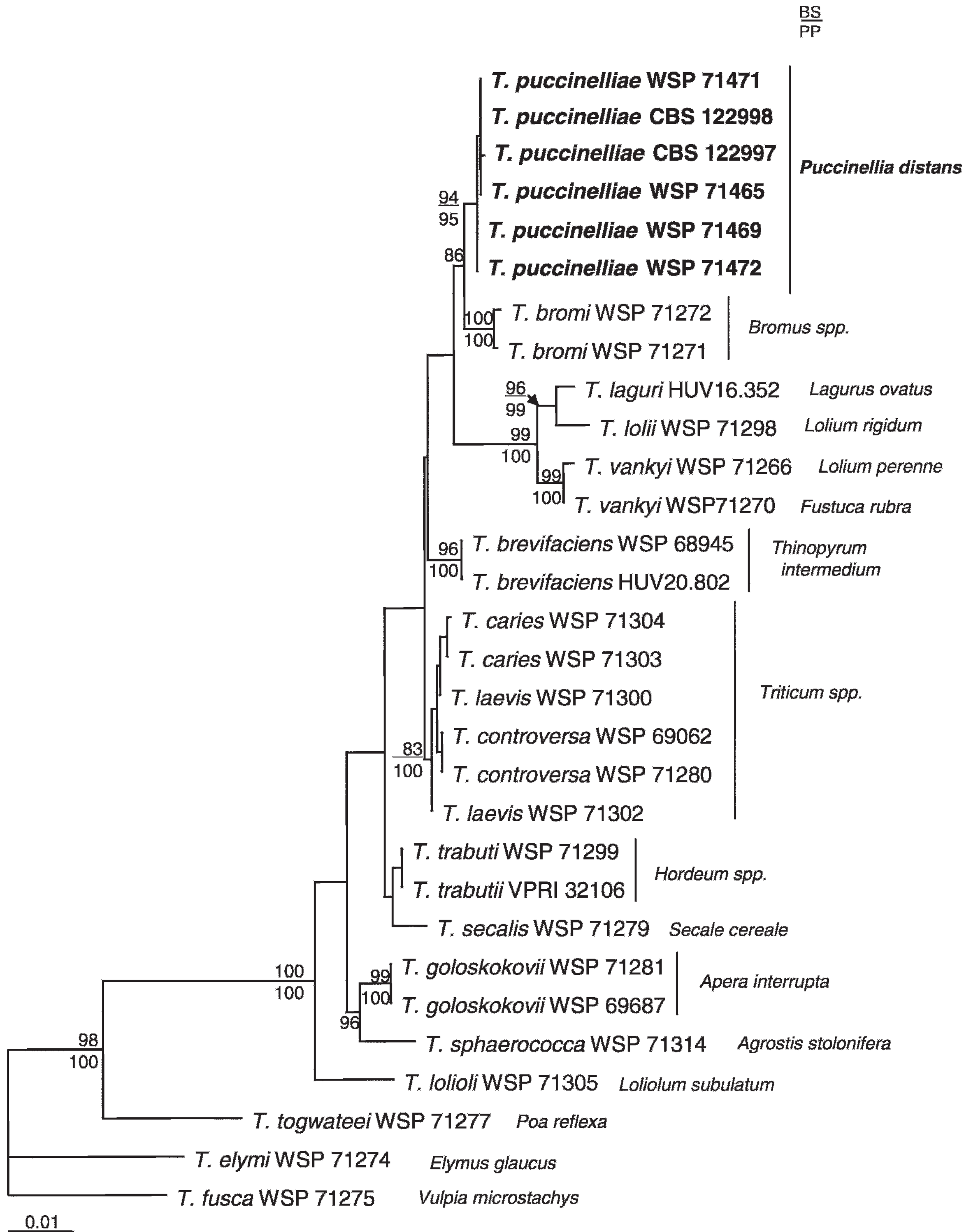

FIG. 4. Maximum likelihood tree of 17 *Tilletia* spp. with the combination of EF1A, ITS1 and RPB2 genes, unrooted. Branch length indicates the substitution rate. Maximum likelihood bootstrap (BS) values above 80% are indicated on branches, and Bayesian posterior probabilities (PP) values above 95% are indicated under branches.

infects (e.g. systemically or locally through developing florets) is not known. However *T. puccinelliae* is closely related to other systemically infecting bunts with hosts in the grass subfamily Pooideae, and it likely infects at the seedling stage and grows systemically within the host as do other bunts in this group. The replacement of the seed with teliospores and the presence of several bunted florets attached to the rachis (FIG. 1b) support the systemic nature of this bunt; local infection through the developing floret typically results in a partially bunted seed, and typically only a few florets of the inflorescence are bunted (Carris et al. 2007). We know little about the ability of systemically infecting smuts to survive year to year in perennial grass hosts such as *P. distans* because most of the well studied *Tilletia* spp. infect annual grasses. In several regards *T. puccinelliae* is similar to *T. vankyi*, a recently described bunt fungus infecting cultivated *Festuca rubra* (fine fescue) and *Lolium perenne* (perennial ryegrass) (Carris et al. 2007). Both species were intercepted as bunted florets in turfgrass seed by scientists at the Tianjin Entry-Exit Quarantine and Inspection Bureau, and the mode of infection is not known with certainty for either species. Bunted florets of *T. vankyi* found in seedlots of fine fescue and perennial ryegrass were filled with teliospores, as was the case with *T. puccinelliae*. Greenhouse studies conducted with *T. vankyi* demonstrated that inoculation methods appropriate for both seedling- and floret-infecting species resulted in infection in *L. perenne* and *L. perenne* var. *multiflorum* (annual ryegrass) (Carris et al. 2007).

Tilletia spp. on other hosts in grass tribe Poeae (*Lolium*, *Festuca*, *Loliolum*, *Vulpia* and *Poa*) do not form a monophyletic group based on the multigene analysis in Carris et al. (2007). *Tilletia puccinelliae* isolates form a well supported group in a clade with *T. bromi*, a species restricted to *Bromus* (tribe Bromeae). *Bromus tectorum* was introduced into North America in the late 1800s from Eurasia and has become one of the most abundant plant species in the Intermountain West (Mack 1984). *Bromus tectorum* infected by *T. bromi* is common in and around cultivated grass and grain fields in Washington, and bunted florets of *B. tectorum* occur as contaminants in wheat and grass seed. The spore germination, size, ornamentation and color of teliospores of *T. puccinelliae* and *T. bromi* overlap sufficiently (TABLE II; FIG. 2b, c, h, i) for the two taxa to be considered conspecific in the absence of molecular data, although the irregular shape of *T. puccinelliae* teliospores is distinct from *T. bromi*. Studies using a molecular approach have demonstrated in general that *Tilletia* spp. have a relatively narrow host range usually restricted to a single genus or in some cases to a single host species (Boyd and Carris 1997, Pimentel et al. 1998, Carris et al. 2007). An apparent exception to the narrow host range is *T. vankyi*, which infects *Lolium* and *Festuca* (Carris et al. 2007). However *Festuca* is a large, complex genus shown to encompass species of *Lolium* in phylogenetic reconstructions (Catalán et al. 2004).

When genus *Tilletia* was monographed (Durán and Fischer 1961) 76 species were accepted from among 200 described species. Since Durán and Fischer (1961) the number of species recognized has more than doubled, with 85 new species or new combinations accepted based on the MycoBank database (http://www.mycobank.org). Only 14 of the new species were described from North America, with 10 of these from Mexico described in Durán (1987). As with many other fungi, the known distribution of *Tilletia* spp. clearly reflects the activities of collectors; the majority (52) of new *Tilletia* species have been described by Kalmán Vánky and various coauthors.

Tilletia puccinelliae is one of four new species, with *T. laguri*, *T. vankyi*, and *T. walkeri*, first encountered by seed inspectors and/or quarantine scientists; none of these species has been observed directly on its respective host(s) in the field. The type specimen of *Tilletia laguri* is based on a collection of bunted *Lagurus ovatus* from Italy, which was intercepted in China (Zhang et al. 1995); an earlier collection was intercepted in USA in 1982 in a shipment of dried flowers from Spain, but the specimen (WSP 71282) was not identified as a new species. *Tilletia vankyi*, as previously noted, was intercepted by scientists as the Tianjin Entry-Exit Inspection and Quarantine Bureau in seed of *Festuca rubra* from USA in 2003, and *Lolium perenne* from Australia in 2003, and Germany in 2005 (Carris et al. 2007). The first record of *Tilletia walkeri* was a bunted floret of *L. perenne* from Australia's Kangaroo Valley (New South Wales) found by a seed-testing lab in 1967 and deposited as DAR 16719 as ''*Tilletia* sp.'', although the species was not formally described until it was found in USA 30 y later during the National Karnal Bunt Survey (Castlebury and Carris 1999). These examples illustrate the need for more extensive surveys of cultivated grasses, particularly those being exported as seed around the world.

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